UNIVERSIDADE DO ALGARVE

Faculdade Ciências e Tecnologia

ENHANCEMENT OF SPERMIATION IN MEAGRE (*Argyrosomus regius*) USING TWO ADMINISTRATION METHODS OF GnRHa.

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Master in Aquaculture and Fisheries

University of Algarve

2017

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Dissertation

Master in Aquaculture and Fisheries

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- Declaração da autoria do trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e contsam na listagem de referencia incluída.

Acknowledgements

Hosted and supported by the Hellenic Center for Marine Research (HCMR), Aquaculture institute.

Supported by the COST Association (Food and Agriculture COST Action FA1205: AQUAGAMETE)

SUMMARY

Reared – produced meagre (*Argyrosomus regius*) males (n=15) with 4 years old were monitored and sampled, during the reproductive season for spermiation, collecting sperm and blood 5 times on May 2016. The fish were treated with gonadotropin-releasing hormone agonist (GnRHa) injection and implant (EVAc). The fish were randomly allocated in three treatments, named Control (n=5), Injection (n=5) and Implant (EVAc) (n=5). The experiment on the enhancement of sperm production was performed using GnRHa injection and implant at 15 and 50 μg GnRHa mean weight kg^{-1} dose respectively. During this time the fish were sampling at days 0, 2, 5, 12 and 19 after treatment and sperm and blood were collected. Sperm samples were evaluated for the sperm condition (arbitrary scale), initial sperm motility (min), motility percentage (%), spermatozoa density (cell/ml) and survival at 4ºC using the common method of analysis for sperm quality and the Computer-Assisted Sperm Analysis (CASA), a system that analyses and track each spermatozoa movement. The collected blood was centrifuged to separate the plasma in order to identify the steroid hormones testosterone (T), 11-ketotestosterone (11-KT) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) using ELISA protocol. One fish per treatment was killed for histologic analysis in the last day of experiment. The obtained data from the common analysis of the sperm, CASA and the hormones were evaluated using a two-way ANOVA, with time (0, 2, 5, 12, and 19 Days) and treatment (Control, Injection and Implant) as the factors. GnRHa injection showed a short period of 2 days of effect regarding sperm production enhancement in meagre, while GnRHa implant proved to enhance the sperm production for a prolonged time. The results obtained for fish treated with GnRHa did not present statistical differences regarding the sperm quality parameters analysed both using the common methods of analysis and CASA. Furthermore, testosterone was the major steroid influencing spermiation. In conclusion, GnRHa implants seemed to be more appropriate to induce spermiation in meagre. Analysis of sperm should be conducted with accurate methods such as CASA.

(Key words: Broodstock, Reproductive Season, Spermatogenesis, Spermiation, Endocrine control, Steroid hormones, Gonadotropin Releasing Hormone (GnRH)).

SUMÁRIO

Um stock de corvinas (*Argyrosomus regius*) machos (n=15) de 4 anos de idade foi monitorizado, sendo os indivíduos amostrados durante a estação de reprodução. Durante este período foram recolhidos esperma e sangue 5 vezes durante o mês de Maio de 2016. Os indivíduos foram separados em 3 tanques, sendo 2 deles tratados com injeções de gonadotrofina (GnRHa, n=5) e implantes de GnRHa (EVAc, n=5). Um tanque foi deixado para controlo da experiência (sem injeção, n=5). A produção seminal foi estimulada através de injeções e implantes de doses de 15 e 50 µg GnRHa por média de peso kg^{-1}, respetivamente. Durante esse período, os peixes foram amostrados nos dias 0, 2, 5, 12 e 19 depois de tratados com GnRHa, recolhendo-se amostras de esperma e sangue. As amostras de esperma foram avaliadas segundo os seguintes parâmetros: condição do esperma (escala arbitraria), mobilidade (min) e percentagem de espermatozoides móveis (%), densidade (células/ml), sobrevivência do esperma armazenado a 4ºC e *Computer- Assisted Sperm Analysis* (CASA). As amostras de sangue foram preparadas para a determinação das seguintes hormonas esteroides: testosterona (T), 11-ketotestosterona (11-KT) e 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) através do método de ELISA. No final da experiência, foram realizadas análises histológicas às gonadas para confirmar os estados de desenvolvimento gonadal. Os resultados obtidos com as análises comuns do esperma, CASA e determinações hormonais foram submetidos a análise estatística (ANOVA de duas vias), com o tempo (0, 2, 5, 12 e 19 dias) e tratamentos (controlo, injeção e EVAc) como fatores. Observou-se que os indivíduos tratados com injeção de GnRHa apresentaram um curto efeito (2 dias) em relação a produção de esperma, enquanto que os indivíduos tratados com implantes (EVAc) sofreram um prolongamento na produção de esperma durante todo o período amostrado. Os resultados para as análises comuns e com CASA revelaram que o esperma de corvina não apresentou diferenças estatísticas em relação aos parâmetros de qualidade. Em relação aos níveis de testosterona determinados, verificou-se que esta hormona atuou como a principal hormona influenciando a espermiação. Em conclusão, os implantes de GnRHa parecem ser o tratamento mais apropriado para a indução da espermiação na corvina. A análise do sémen deve ser realizada utilizando métodos precisos, tais como o CASA.

(Palavras-chave: Reprodutores, Temporada Reprodutiva, Espermatogênese, Espermiação, Controlo Endócrino, Hormônios Esteróides, GnRH)).

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1 INTRODUCTION

Aquaculture

Aquatic species under domestication efforts are rising rapidly as a result of a commercial aquaculture development in the past years (Donaldson, 1996). The global fish consumption has been supported by aquaculture. Aquaculture industry becomes the most rapidly growing food sector, since the 1970s it has grown at more than 8% annually. Fishery and aquaculture operations act as an important source of food security and also help in livelihoods of millions of people special in developing countries (FAO, 2014). However the consumption and demand for fish and shellfish are increasing, the fisheries sector is stagnant or decreasing. The intense fisheries with no concern about environmental and biological aspects required for each species leads to a necessity of improvement in the aquaculture field in order to decrease the over demand from the oceans (FAO, 2014). Therefore the aquaculture sectors are becoming more important, once that can provide social, economic and more sustainable resources (Edwards, 2015).

The practice of aquaculture is very old in Asian countries, China has an aquaculture history dating back more than 2500 year and it is the leader now a days of a massive production of fresh water fish, seaweed and marine fishes. Europe starts to have a considerable production on 80´s, and today the aquaculture sector accounts for about 20 % of fish production. The EU aquaculture is distinguished for its high quality, consumer protection and sustainability. However, the potential for large-scale production faces some challenges, such as solutions offering long-term environmental, consumer acceptance of farmed products, economic and social sustainability (FAO, 2014). The capacity to control reproductive process of fish in captivity is an important goal for the aquaculture companies (Mylonas and Zohar, 2009). The selection of species with biological and economical potential may enhance aquaculture production. Several researches on emerging species with great potential for aquaculture have been done in order to solve production bottlenecks, increase knowledge and technology and offer new products to the market [Babiak *et al.*, 2006; Bentley *et al.*, 2009; Zohar and Mylonas, 2001). New species with good aquaculture potential are emerging in attempt to increase the European aquaculture sector and as an alternative to decrease the fish capture, such species are: greater amberjack (*Seriola dumerili*), wreckfish (*Polyprion americanus*), Atlantic halibut (*Hippoglossus hippoglossus*), grey mullet (*Mugil*

cephalus) and pikeperch (*Sander lucioperca*), shi drum (*Umbrina cirrosa)* and meagre (*Argyrosomus regius)* (Diversify III, 2014).

Meagre (*Argyrosomus regius*) is one of the species candidates that shows a high aquaculture potential due to some specific characteristics such as relatively easy broodstock management; easy larvae rearing with standard industry live feeds and formulated diets; and the juveniles that do not present reproductive maturation during ongrowing (Mylonas *et al*., 2013b). In addition and very important, the species achieves a large size in a short period of time, with growth of ~ 1 kg per year and low feed conversion ratio of 0.9 – 1.2. Another characteristic is the flesh that has good processing yield, low fat content and excellent taste and firm texture (Manford, 2010). Despite the fact that meagre shows a great potential for aquaculture, the most important bottleneck regarding meagre rearing is related to reproduction and larval rearing (Battaglene, 1994). Therefore, to achieve success in reproduction in captivity is essential to understand the species reproductive strategies and maturational cycle, i.e. sexual differentiation, size and age at first maturity, reproduction dietary requirements, maturational development in relation with environmental changes, reproductive endocrinology, spawning behaviour and egg parameters. Knowledge in reproductive strategies and maturational cycle provides conditions to achieve the late stages of gametogenesis, oocyte maturation and spermiation in aquaculture (Mylonas *et al.*, 2010).

Distribution, habitat feed and reproductive features of meagre

The meagre distribution covers Norway to Senegal along the Atlantic coast including the Mediterranean Sea (Abou Shabana *et al*., 2012) and Black Sea (Cárdenas, 2011). The populations of wild meagre in the Mediterranean waters have suffered alarming declines and have disappeared from some areas, such as the Balearic Islands, where they are considered to be critically endangered (Gil *et al.*, 2013). Meagre is a carnivorous species that feeds on polychaetes, echinoderms, mollusks, crustaceans and small fish species. It is semi pelagic specie found on rocky coastal areas and also in Posidonia fields or close to small mullets and sardines that are part of their diet. Is a gregarious species with nocturne habits moving in small groups around rivers and lagoons estuaries out fall during spring and summer (Cárdenas, 2011; González-Quirós *et al.*, 2011). At the reproductive season between April and July they change behaviour

and start to move in big groups for spawning, estuaries areas are used for the juveniles development (Cárdenas, 2011; Mylonas *et al.*, 2013b). There are other studies where the reproductive season of meagre ranged between March – June in Spain (Cárdenas, 2011; Gil *et al*., 2013), June and July in Italy (Schiavone *et al.*, 2012) and between April and August in the Gulf of Cádiz, Spain (González-Quirós *et al.*, 2011). The differences in the reproductive season can be related with the water temperature (Mylonas *et al.* 2016b). During the spawning season meagre males produce a typical sound by impelling their abdominal muscle against the swim bladder (Lagardère and Mariani, 2006). The species remain in the estuaries areas until July, the main factor for the reproductive migration may be related with water temperature (between 17ºC and 21ºC) and consequently increase metabolism. The food consumption and growth rate during the spawning season increase, while during autumn and winter the adult individuals go to deep waters at temperatures lower than 13 and 15ºC and decreases their feed activity (Cárdenas, 2011; González-Quirós *et al.*, 2011). This specie is classified as a gonochoristic specie that reaches puberty at 2 and 3 years of age for males and females respectively, and exhibits an asynchronous or group – synchronous gonadal maturation development which means that they spawn multiple times along the reproductive season (Schiavone *et al.*, 2012). The first maturation season in nature occurs at a total length (TL) of 45 cm – 62cm for males and 47 cm -70 cm for females (González-Quirós *et al.*, 2011). In captivity the TL reported for maturation is smaller, at 27 cm (2 years old) for males and 36 cm (3 years old) for females (Schiavone *et al.*, 2012).

Reproduction

The fish reproductive cycle is a process that involves the development of germ cells to form mature gametes. The germ cells develops and proliferate during the gametogenesis (vitellogenesis and spermatogenesis), and became mature during the oocyte maturation and spermiation. It is in the maturation phase where the oocyte and spermatozoa acquire the capacity to be release and fertile (Schulz *et al*., 2010).

Spermatogenesis and spermiation

Gametogenesis in males is separated in three phases (Figure 1).The spermatogenesis is the first phase, spermiogenesis the second phase and spermiation is the phase three. In the first phase the spermatogonia germ cells A proliferate and differentiate in spermatogonia B, which perform multiple mitotic divisions being genetically

determined and specie-specific, resulting in late spermatogonia B. In the spermiogenesis, the spermatogonia B goes to meiotic divisions and become spermatocytes I and II which will turn in spermatids. The last phase (spermiation), the spermatids differentiate in flagellated spermatozoa becoming able to move and to fertilize (Mylonas *et al.*, 2016a). This processes is defined by the release of the spermatozoa in the sperm ducts, which cause the hidration of the testes because of the production of seminal fluid and changes in the pH. This fluid containing spermatozoa may be collected by stripping. However, in some cases the stripping can be difficult due to anatomical reasons (Schulz *et al.*, 2010).

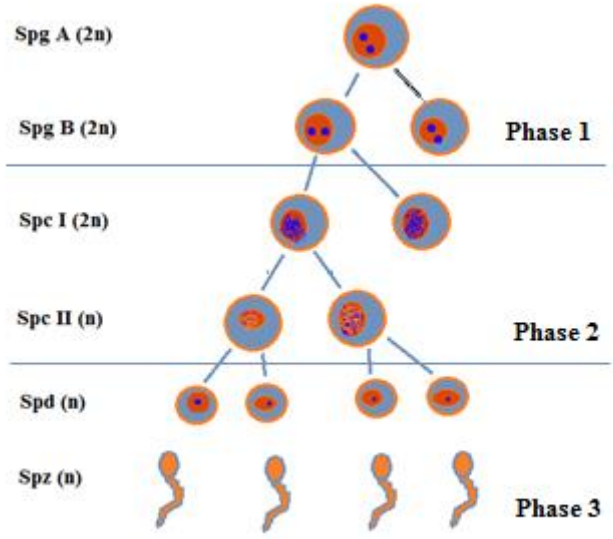

Figure 1. Gametogenesis in male fish, spermatogenesis, spermiogeneses and spermiation.

Testes development

The development of the testes has many different characteristics between the invertebrates. Among the fish, the structural morphology and the process thorough developmental stages of the germ cells constitute the main differences. The differences in the testes development in fish species are related to: a) the time that the germ cells take to develop in relation with the seasonality of the spawning season of the fish species (synchronous, asynchronous), b) the testes structure (tubular or lobule) and the migration of the spermatocytes to the lumen (restricted or unrestricted), c) the stage of

development that the germ cells leave the spermatocyte (cystic or semicystic) (Mylonas *et al.,* 2016a ; Schulz *et al.,* 2010).

Important species such as sea bass (*Dicentrarchus labrax*), (Mañanós *et al.,* 2002) spotted rose snapper (*Lutjanus guttatus*) (Mylonas *et al.,* 2016a) and meagre (*Argyrosomus regius*) present an unrestricted lobular testis. The shape of the testes and unrestricted type means that the development of the different stages of germ cells occurs random within the testis, with no restricted areas. However this differences in the testes structure does not affect the sperm production (Mylonas *et al.,* 2016a ; Uribe *et al.,* 2014).

Endocrine regulations in fish

Spermatogenesis and spermiogenesis are regulated by the axis; brain- pituitary-gonad. The brain reacts from external information such as temperature, day light, salinity, social interaction, and stimulates the action of the gonadotropins-release-hormones (GnRHs) in the pituitary. The synthesis and release of gonadotropin Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) are controlled mainly by the action of this GnRHs (Schulz *et al.,* 2010). However some fish present a dopamine inhibition of the stimulatory effect of GnRH, especially fresh water fish (Peter and Yu, 1997). Recently other hormone Kisspeptin has been shown to influence GnRH and expressions of FSH and LH in the pituitary (Zmora *et al.,* 2015).

The hormonal regulation of fish spermatogenesis and spermiation is moderate by the FHS and LH released into the bloodstream to act directly on the gonads with the production of androgens, estrogen and progestins (Figure 2). The FHS essentially is involved in the spermatogenesis through the synthesis of androgens, testosterone (T) and 11 keto-testosterone (11-KT) and other growth factors in the Leydig cells and the Sertoli cells. The LH stimulates the shift to start the synthesis of maturation inducing steroid (MIS) (Mylonas *et al.,* 2010 ; Mylonas *et al.,* 2016a).

Before starts the reproductive season, the fish gonads are regulated by the hormone estrogen (E_2). When the spermatogenesis starts, the germ cells starts to proliferate and become spermatogonia regulated mainly by 11-KT, the levels of androgens steroids increase during spermatogenesis and decrease in spermiation. The spermiation is controlled by the LH in the Leydig cells which promotes a steroidogenic pathway that

induces the production of MIS. The MIS is a progesterone synthesized in the spermatozoa, MIS can be different depending on the specie; 17,20β- dihydroxy-4-pregnen-3-one (17,20β-P) or 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P or 20β-S). In the spermiation, the MIS cause changes in the PH and increased of seminal fluid which is very important for the spermatozoa to acquire the capacity to fertilize and move forward (Mylonas *et al.*, 2010; Schulz *et al.*, 2010).

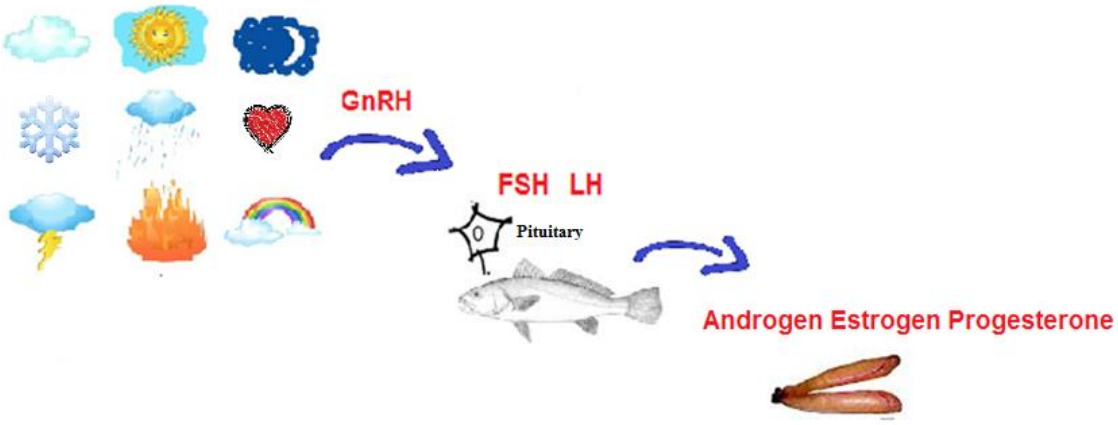

Figure 2. Fish endocrine regulation

Problems regarding fish reproduction in captivity

Reproduction problems are observed in many species well-establish in aquaculture and also new ones with high commercial interest. Despite the fact that most of the studies regarding reproduction dysfunctions are pointed to females, control of sperm production and quality are becoming a very important subject for the aquaculture industry (Cabrita *et al.*, 2014). The most common problem of male fish in captivity is regarding the poor or reduced amount of milt production and or viscous milt which prevent the dispersal of the spermatozoa in the water. Moreover the spermatozoa became active after contact with water and remain active for just a few seconds or minutes. Low LH plasma levels in the brain or problems with GtH synthesis during spermiation have been suggested as the origin of these problems (Mylonas *et al.*, 2010). Some species like the males Japanese and European ell exhibit testes containing just spermatogonia and fail to produce sperm, to continuous the spermatogenesis and reach the spermiation its necessary to apply a long term hormonal treatment. This treatment promotes the increase of 11-KT which achieve spermatogenesis and spermiation (Mylonas *et al.*, 2016a; Schulz *et al.*, 2010).

Broodstock management concerning problems in reproduction

The most important factors for successes reproduction in captive are the identification of the optimal condition necessary for the species to undergo reproductive maturation and produce good quality of gametes (Mylonas and Zohar, 2009). The environmental factors such as photoperiod, temperature, salinity, pH, social interaction influence the spermatogenesis and spermiation and the ability of the fish to spawn and produce good quality sperm. Furthermore the nutrition and stress are considered also limiting factors for reproduction. Therefore it is necessary to adjust the environmental parameters that can be controlled to the culture systems, to ensure the best condition for the fish and ensure good quality sperm (Alavi and Cosson, 2005; Mylonas *et al.*, 2016a; Pavlov, 2006)

Hormonal treatment to enhance poorly spermiation in fish

The methods to enhance spermiation were based in the use of exogenous LH to act directly in the gonads and induce the spermiation, once that the failure in spermiation can be explained by the lack of this hormone during the spawning season. Also the use of gonadotropin release hormone (GnRHa) (with dopamine or without) that act in the pituitary but release the endogenous LH stored. However, the GnRHa can induce as well the process of spermatogenesis, spermiogenesis and spermiation because it releases the stored hormones in the pituitary (FHS and LH) (Mylonas *et al.*, 2016a; Schulz *et al.*, 2010)

Many hormonal treatments aiming ovulation and spermiation have been tried even before the exact understanding of the endocrine controls of the fish. Exogenous LH preparations from the pituitary of mature carp or salmonids in the reproductive season (CPE or SPE) were the first hormonal treatment used in aquaculture (Von Ihering, 1937). This method has the advantage to be cheap and easy to prepare, induces gametogenesis, ovulation and spermiation fast and direct in the gonads, once that the pituitaries are extract in the reproductive season and contain mainly LH but also FSH. However, it presents some disadvantages too, e.g. It is difficult to calculate the correct hormonal dose per fish, because it depends on the donor, the sex and the reproductive stage of the fish, also the exogenous pituitary contain different compounds that can cause immunoreactions and transmit diseases to the broodstock. PE is commonly used, especially in developing countries, where the access to other methods is expensive

(Mylonas *et al.,* 2010; 2016a). Other methods like recombinant GtHs from fish or mammalians origins are successful used in some aquaculture species. However the most common problem regarding all these methods is the short effect lived (Mylonas *et al.*, 2016a), the consecutive handling required and timing consume, which cause stress and can affect the gametogenesis and spermiation (Schreck, 2010).

GnRHa injections and delivery systems

The use of GnRHa increased fast due to the advantage that offers when compared with LH preparations. Treatments with GnRHa are less specie specific because of it similarity with the native GnRH from the fish, also the synthetic nature prevent the transmission of diseases and allows a better calculation of an effective dose. Additionally the GnRHa treatments act in the level of the brain-pituitary-gonads and stimulate the release of endogenous LH and FSH and other hormones involved in the reproduction (Mylonas *et al.*, 2010). Also, due to the low molecular weight is easily to incorporate in control-release systems. Therefore different forms of GnRHa delivery systems have been used widely. The current delivery systems available in the market can be in solid implantable pellets of cholesterol, Ethylene Acetate (EVAc), biodegradable microspheres injectable, etc (Mylonas and Zohar, 2009). Oocyte maturation and spermiation require a long-term hormone treatment and a single injection of GnRHa may present a short effect in the reproductive function. In the golden rabbitfish (*Siganus guttatus*) one injection increase the sperm volume for 24 hours but after 48 hours of treatment the spermiation was similar to the non-treated fish (Garcia, 1991; Komatsu *et al*., 2006). Even though the GnRHa injection can be administrated to fish with different sizes without changes in the hormonal preparations, the delivery system offer less accuracy in the treatment due to the different size of the fish. GnRHa delivery systems promote a long term treatment; can release hormones for 1 to 5 weeks, depending on the matrix and water temperature (Mylonas *et al.*, 1997; 2016a). In European sea bass, GnRHa implants enhance spermiation for at least 27 days in contrast with GnRHa injection that stimulates spermiation for 3 days. (Rainis *et al.*, 2003).

Fish sperm quality

Aquaculture is dependent on the spawning of fish in captivity where the conditions may be very different from their natural habitat. Control of sperm quality is an important

variable that contribute to the oocyte fertilization and the success of the embryonic development (Kime *et al.*, 2001). Furthermore it is also important for the development of methods and protocols of sperm storage and cryopreservation that secures a good quality. The quality of the sperm require a consistent understanding of the mechanisms involved in the male breeder performance and biological characteristic of the specie and most important the broostock conditions e.g. nutrition, environmental manipulation and management (temperature, pH, social interactions, pherormones, sex ratio), spawning induction protocols (Cabrita *et al.*, 2014). Different parameters have been used to evaluate sperm quality e.g sperm volume, sperm concentration, sperm motility in minutes and percentage, sperm velocity, sperm morphology and seminal plasma composition (Mylonas *et al.*, 2016a). The most common characteristics of sperm quality assessed by easy methods are: sperm volume, density and sperm motility minutes and percentage. The sperm motility assessed by microscope is considered a subjective assessment, once that rely on the judgment of the person who is analysing. Even though has been so far widely used for qualify spermatozoa quality, specially before fertilization trials (Fauvel *et al.*, 2010). However, nowadays new technologies have been performed aiming ensure a better evaluation of sperm quality with more accuracy, such as Automated Sperm Morphology Analysis (ASMA) for sperm morphometric features and Computer- Assisted Sperm Analysis (CASA) to assess sperm motility parameters (Cabrita *et al.*, 2014; Cosson *et al*., 2008).

2 OBJECTIVE

The current study aim to test two methods of GnRHa treatments, injection and implant on meagre (*Argyrosomus regius*) in order to identify the best hormonal therapy induction to enhances the sperm production in this fish species. With this, it is expected to achieve a better understanding of GnRHa effects on the endocrine system of this fish which will help to develop further protocols for hormonal treatments. Furthermore, it was intend to qualify the sperm characteristics and evaluate the effect of the GnRHa treatments in the quality of the sperm, using extensively used methods and a new program CASA for assessment of sperm motility. The information obtained from the experiment analysis is expected to be useful for enhance sperm production, improve the reproduction success of meagre in captivity and develop methods to standard protocols that can be useful for commercial aquacultures.

3. MATERIALS AND METHODS

3.1 Broodstock maintenance

A stock of 4 years old meagre n = 26 (mean ±SD weight of 3.91 ± 1.16 kg) were undertaking at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (HCMR), in Iraklion. The fish were maintained in rectangle fibre tank with 10.04m³volume, water flow supplied with aerated seawater and food was given five days per week. Fish were feed daily with a commercial pellet until apparent satiety and starved for 2 days before sampling to avoid contamination. The fish were maintained in a tank exposed to simulate natural thermo-conditions (21ºC), and were individually distinguished with a PIT tag (AVID, UK). The measurements of pH, dissolved oxygen (% saturation), nitrites and ammonia were taken every week.

3.2 Evaluation of reproductive stage and broodstock selection

Apparently the meagre broodstock were starting their first spawning season. The broodstock was sampling once per month since February 2016, in order to monitor the spermiation condition. The experiment with GnRHa treatment was set to start when most of the fish reach at least some stage of spermiation, in the beginning of the spawning season. The spawning season for meagre reared in this temperature (21ºC) normally starts in April lasting until July (Mylonas *et al.*, 2013b). The first sampling to select the fish (n=15) for the experiment started on 25 of April 2016. The fish were selected according to their spermiation condition, determined by a subjective scale: S0 = do not release sperm after stripping, S1= only a few drops of sperm after several stripping's, S2= release some amount of sperm after the first stripping and S3= release sperm easily (Mylonas *et al.*, 2013a). The sperm was expressed out from the testes after application of gentle abdominal pressure.

3.3 Treatment with GnRHa injection and EVAc implants

The experiment with gonadotropin release hormone analogue (GnRHa) started on 4 of May 2016 as day 0 of experiment and finished 23 of May as Day 19, within the days of experiment the fish were sampled during the Day 2, Day 5, Day 12 and Day 19. The first day of experiment – Day 0, the previous selected fish (n=15) were divided into 3 groups (n = 5) and placed in separate tanks as follows: control (C), injection (Inj) and implant (EVAc). The fish from the Control (Cntrl) group were pinched with a syringe to

simulate the procedure of an injection or implant, fish from Injection group (Inj) were injected with 15 µg mean weight kg^{-1} dose of GnRHa and the group of fish EVAc were implanted with Ethylene-vinyl Acetate copolymer (EVAc) at a dose 50 µg GnRHa mean weight kg^{-1} loaded with Des-Gly ^{10}, D-Ala6-Pro-NEth9-mGnRHa (H-4070, Bachem, Switzerland) (Mylonas *et al.,* 2015). The following samplings after Day 0 had the same procedure, except for the Day 12 were was applied Inejections of GnRHa again in the group injected at a dose of 15 µg GnRH mean weight kg^{-1} (Fernandez-Palacios *et al.,* 2014).

3.4 Sampling procedure

The water flow was reduced until safety level and the fish tranquilized in their tank with clove oil (0.01 ml 1-1 of clove oil dissolved 1:10 in 96% Et OH) and transferred to a small tank for complete sedation with a higher concentration of clove oil (0.03 ml 1-1). The fish were identified from their PIT- tag, stripped to evaluate their spermiation condition (S0, S1, S2 and S3) and to collect the expressed milt. To collect the sperm, the genital pore was carefully wash with water and dried in attempt to avoid the sperm contamination with urine or faeces and with a displacement dispositive pipette (10- 100 µl) the expressed milt was collect, add in a hematrocit tube and stored on ice for posterior sperm quality evaluations. To check the level of hormonal steroids presented in the fish, 2.5 ml of blood was taken with a heparinized syringe introduced in the pelvic peduncle of the fish, towards the vertebral column until find the artery and bump up the blood to the syringe, the blood was stored in tubes with 125 µl of anticoagulant perfablock and kept on the ice until be centrifuge. Afterward the fish were allocated in the tank in the same conditions as before.

Figure 3. Sperm collection during the first day of experiment, Day 0.

3.5 Sperm analysis

3.5.1 Evaluation of spermatozoa

Immediately after the sampling the evaluation of spermatozoa motility duration (min), percentage (%) and density was performed. The motility duration was measured in duplicate with a microscope and a chronometer, by the time immediately after activate the sperm with of sea water mixed with Bovine Serum Albumin (BSA) at a concentration of 1:10, until the cells present < 5% of flagellar movement. The sperm was activated with a drop of seawater (from the fish tank at the same temperature, 21ºC mixed with BSA (1:10) in a slide glass and with a few amount of sperm add on the glass with a positive displacement pipette. The initial sperm motility percentage also was evaluated in duplicates after activation, with the same preparation of seawater and BSA. After evaluated, the sperm was stored at 4ºC and in order to determine survival at short storage, the motility percentage was measured after one day of sampling, 3, 5 and 7 days, continuously until the sperm shows no percentage of motility.

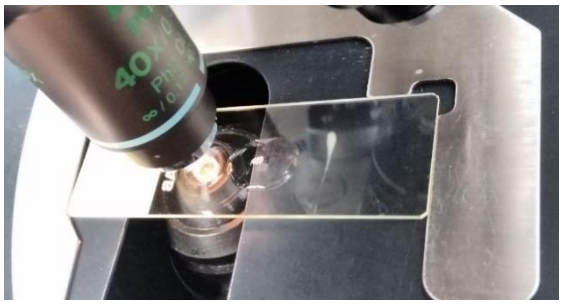

Figure 4. Meagre sperm activation with seawater and BSA on the microscope.

To obtain an appropriate concentration for counting the density of spermatozoa cells, the sperm was diluted in 10: 200 µl (sperm saline solution) and then a second dilution 10:1000 µl, totalizing 2121 µl dilution and added in the Neubauer hemocytometer, counted 5 squares in duplicates, the upper and down part of the hemocytometer under 40 x microscope magnification.

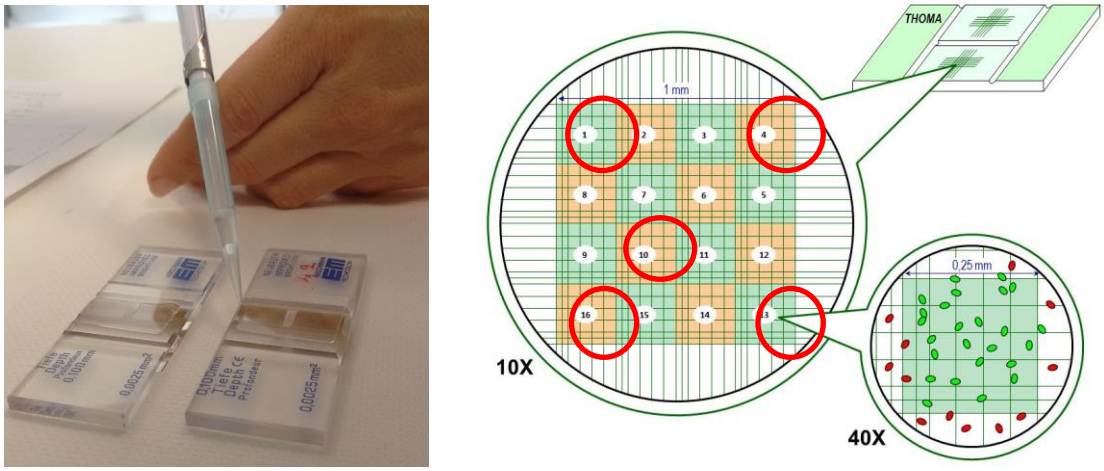

Figure 5. Haemocytometer glass used to count the meagre sperm density.

3.6 CASA sperm analysis

In order to improve the methodology of assessing sperm quality and to make it objective and quantitative Computer-assisted sperm analysis (CASA) was performed. The analysis involves videotaping the sperm movement via microscope and then using the plugin CASA with established parameters to replay the record frame by frame and mark the position of each sperm in the video record.

3.6.1 General Description of the CASA method

The process involved the use of four computer programs. To capture the images a video camera (DMK 22BUC03 from Imaging source) was mounted to a microscope connected via USB in a computer and the sperm movement was recorded in a video with IC Capture 2.4 software steed on AVI format, 30 frames per second and zoom 4 x. The frame images were selected with Virtual Dub (virtualdub.org), the image frame sequences were imported and processed with ImageJ (www.uhnresearch.ca/wcif free software) and subsequently analysed with CASA plugin within the ImageJ. As a result an Excel file was opened with the values for motility percentage(%), curvilinear velocity (VCL), velocity average path (VAP), straight line velocity (VSL), linearity

(LIN), wobble (WOB), progression (PROG), beat cross frequency (BCF) and the number of cells analysed in micrometres per second (µm/s).

3.6.2 Sperm preparation

The sperm was video recorded two times per sample: 1) with a non-activating medium (NAM)– milt was diluted 1:3, 50 µl NAM added with a pipette in a 0.5 ml microtube and with a dispositive displacement pipette added 25 µl of sperm. This dilution intent to adjust the concentration of spermatozoa without promoting motility and improves the semen storage. 2) directly with fresh sperm.

To triggering the natural motility of the spermatozoa an activate medium was prepared using 18 ml of seawater from the fish tank (21ºC) supplemented with Bovine Serum Albumin (BSA), a protein that prevent the spermatozoa to be stick on the glass.

3.6.3 Preparation to activate and record

The video camera was install in the microscope with negative objective phase 3 contrast, with magnification 20 x. The Leja chamber was placed in the real position and lightning and focus were adjust before starting to record. For each trial (NAM and fresh sperm) one tube of 1.5 ml was filled with 99 µl of activate medium (seawater with BSA). To start the recordings, 4 - 9 µl of NAM was deposit on the wall of the tube filled with seawater and BSA, with no contact between them. The button to record was pressed immediately when the solutions were mixed, quickly 1 - 3 µl of the dilution was taken from the tube with a pipette and introduced in the Leja chamber, which was already adjust in the microscope ready to receive the sperm. The sperm was record until present last than 5% of motility. To record the fresh sperm, the same procedure was performed, using 1 - 3 µl of sperm on the wall of the tube to mix with activate medium and add on Leja to record.

3.6.4 CASA analysis

After video record, 60 frames were selected (2 seconds) for every 10 seconds of video, starting with the first seconds of initial sperm motility of the video. The program Virtual Dub was used to select the image sequence in frames. Each image sequences (60 frames) were imported to ImageJ, opened as a sequence of stack frames and processed using Image => 8 bit, Image => Adjust => Brightness/Contrast in order to facilitate the choice of particles, Image => Adjust => Threshold to separate the particles of interest

from the background (binarization). As a result it was obtained black particles spots to be studied in a white background. After the ImageJ processes the images were analysed within the plugin CASA resulting in a draw of the cells movement, where the black lines indicated alive cells and the grey the dead ones.

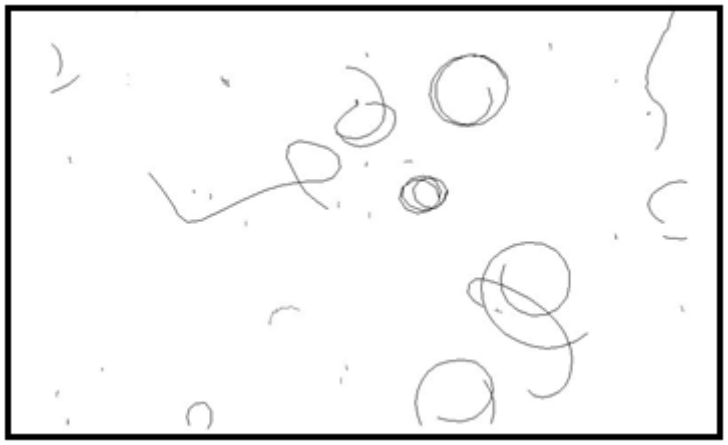

Figure 6. Sperm movement as result of CASA analysis.

3.6.5 CASA and EXCEL

As a result from the plugin CASA in ImajeJ, an Excel file was opened with sperm source of motility percentage %, velocity curvilinear (VCL/µm/s.), velocity average path (VAP/µm/s.), velocity straight line (VSL/µm/s.), linearity (LIN), wobble (WOB), progression (PROG/µm/s.), beat cross frequency (BCF-µm/s.) and the numbers of anazysed cells. However statistical analysis were made using the results of sperm motility (%), VCL VAP and VSL in µm/s.

3.7 Steroid Hormonal measurements

The blood collected was centrifuge in order to separate the plasma from the blood and stored in -80 º C until be analysed with an established enzyme-linked immunoassays (ELISA) (Cuisset *et al*., 1994; Nash *et al*., 2000) for the quantification of testosterone (T), maturation inducing steroid (MIS)17-20ß-dihydroxy-4-pregnen-3-one (17,20bP) and 11 – ketotestosterone (11-KT). In total were 74 blood samples that were centrifuge and separate the plasma in 3 aliquots of 0.5 ml.

3.7.1 ELISA meagre

Hormones extraction

A total of 300 µl of plasma from each sample were added into a glass tube with 3 ml of diethyl ether (1:2) and vortex for 3 minutes and freeze for 10 minutes. The suspension was transferred to a new glass tube and evaporate under nitrogen. The procedure was repeated two times. After finish, 600 µl of reaction buffer (RB) was added in the empty glass tube for the reconstitution of the samples.

To start the assay preparation, the microplates, reaction buffer (RB), standard (Std), antibody (AB) and tracer (TR) required for this technique were already prepared at specific concentrations (appendix 2). Afterwards, the dilutions decided to be used were based on previews experience working with meagre hormones, which does not present high levels of hormones.

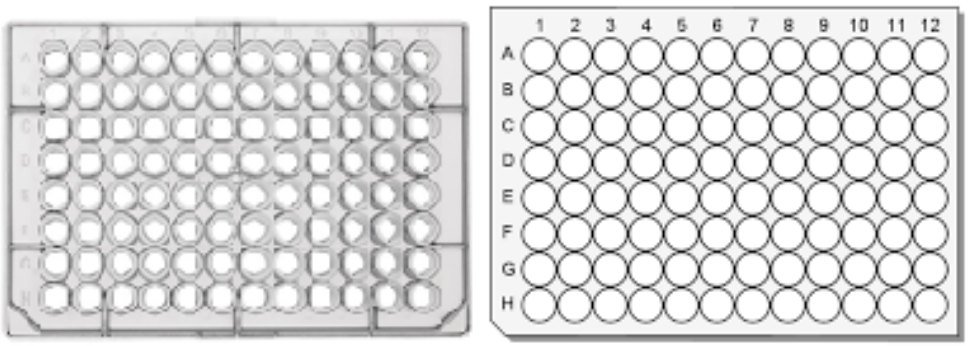

Figure 7. Microplate

3.7.2 TESTOTERONE hormone analysis

The microplates were washed 3 times with a washing buffer in a Microplate Washer. To prepare the testosterone standard (Std) 11 eppendorfs were filled with 150 µl of RB. Therefore 50 µl Std (1 ng/ml) was mixed with 450 µl RB to obtain 500 µl (100 ng/ml). In the first eppendorf (1) 50µl Std (100ng/ml) was added with 950 µl RB and mixed with a vortex machine. The consecutive tubes were diluted taking 150 µl of solution from the previous tube and adding in the next always using the vortex machine before exchange the solutions. The procedure was repeated for all the eppendorf tubes (Figure 8)

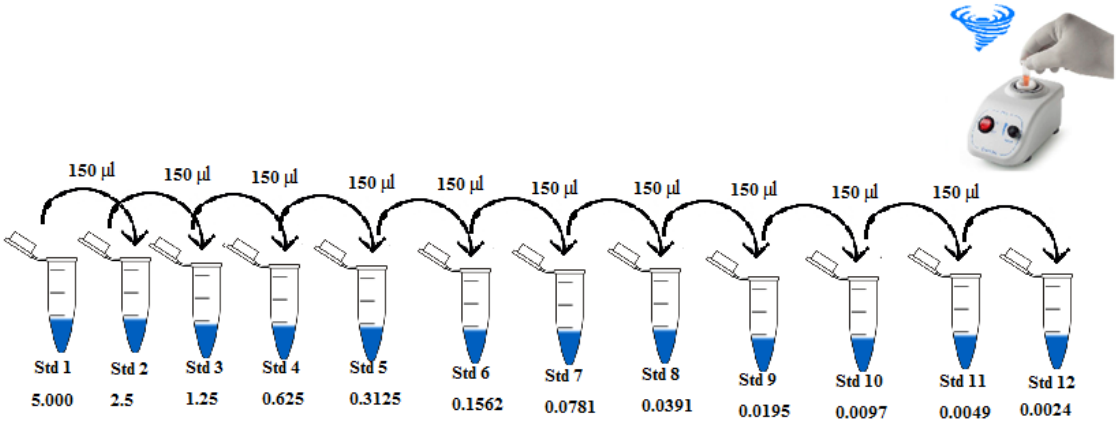

Figure 8. T Standards preparation illustration with each standard dilution in ng/ml.

After the standards were prepared, 270 µl of TR was mixed with 5130 µl RB in a plastic cup in order to obtain 5.4 ml TR. The AB was prepared in the same procedure, mixing 30 µl AB with 5970 µl RB to obtain 6 ml of AB solution.

3.7.3 11-KETOTESTOSTERONE hormone analysis

The microplates were washed 3 times with a washing buffer in a Microplate Washer. To prepare the 11-KT standard (Std) 11 eppendorf were filled with 150 µl of RB. Therefore 50 µl Std (10ng/ml) was mixed with 450 µl RB to obtain 500 µl (1ng/ml) as the first dilution in the eppendorf 1. The consecutive tubes were diluted taking 150 µl of solution from the previous tube and adding in the next and vortex. The procedure was repeated for all the eppendorf tubes (Figure 9).

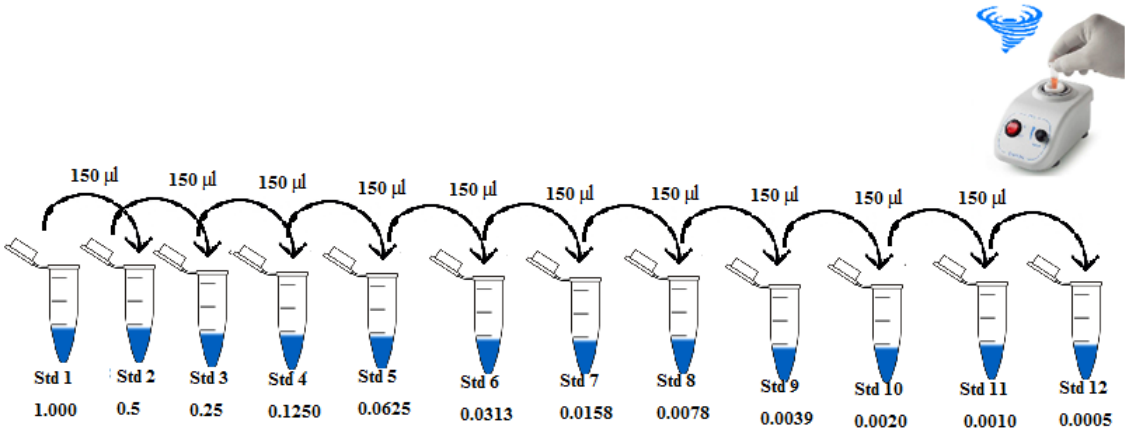

Figure 9. 11-KT Standards preparation illustration with each standard dilution in ng/ml.

After prepare the standard, 1 ml of TR was mixed with 4 ml RB in a plastic cup in order to obtain 5 ml TR. The AB was prepared using the same procedure, 30 µl AB with 5970 µl RB to obtain 6 ml of AB solution.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**N**	**Bo**	**Std 1**	**Std 5**	**Std 9**	**P**	**P**	**P**	**P**	**P**	**P**	**P**
B	**N**	**Bo**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**
C	**N**	**Bo**	**Std 2**	**Std 6**	**Std 10**	**P**	**P**	**P**	**P**	**P**	**P**	**P**
D	**NSB**	**Bo**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**
E	**NSB**	**QCH**	**Std 3**	**Std 7**	**Std 11**	**P**	**P**	**P**	**P**	**P**	**P**	**P**
F	**NSB**	**QCh**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**
G	**AT**	**QCl**	**Std 4**	**Std 8**	**Std 12**	**P**	**P**	**P**	**P**	**P**	**P**	**P**
H	**AT**	**QCl**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**	**"**

Figure 10. Schematic representation of a microplate

Preparation of the microplate for T and 11-KT

The procedure to prepare the microplates for T and 11-KT were the same. The solutions were placed in the wells in the microplates; 50 µl TR+100 µl RB were added in the non-specific binding (NSB), 50 µl TR + 50 µl AB + 50 µl RB added in the maximum binding (B0), 50 µl TR + 50 µl AB + 50 µl Std were added in replicates in the standard wells (Std) and 50 µl TR + 50 µl AB + 50 µl of the samples (P) were add in replicates in the wells (Figure 10)

The microplates were cover with a plastic cover plate and aluminium and incubate for 1 hour at 22 ºC and agitation 140 r.p.m. After the incubation the plates were washed 3 times in the Microplate washerand the Ellman solution was prepared. To prepare the Ellman 1760 µl of pure water was added with 440 µl of Ellman and placed in 200 µl in all the wells and more 5 µl of TR was added in the total activity (AT) wells.

3.7.4 MIS hormone analysis

The microplates were washed 3 times with a washing buffer in a Microplate Washer. To prepare the MIS standard (Std) 12 eppendorfs were filled with 150 µl of RB. Therefore 60 µl Std (100ng/ml) was mixed with 240 µl RB to obtain 300 µl (20ng/ml) as the first dilution in the eppendorf 1. The consecutive tubes were diluted taking 150 µl of solution from the previous tube and adding in the next and vortex. The procedure was repeated for all the eppendorf tubes in order to decrease the concentration (Figure 11).

After prepare the standard, 63 µl of TR was mixed with 4937 µl RB in a plastic cup in order to obtain 5 ml TR. The AB was prepared using the same procedure, but in two steps dilution, in the first one 20 µl AB was added with 380 µl RB to obtain 4 ml of AB solution and from the first solution 10 µl was added with 490 µl RB and obtained 5 ml AB.

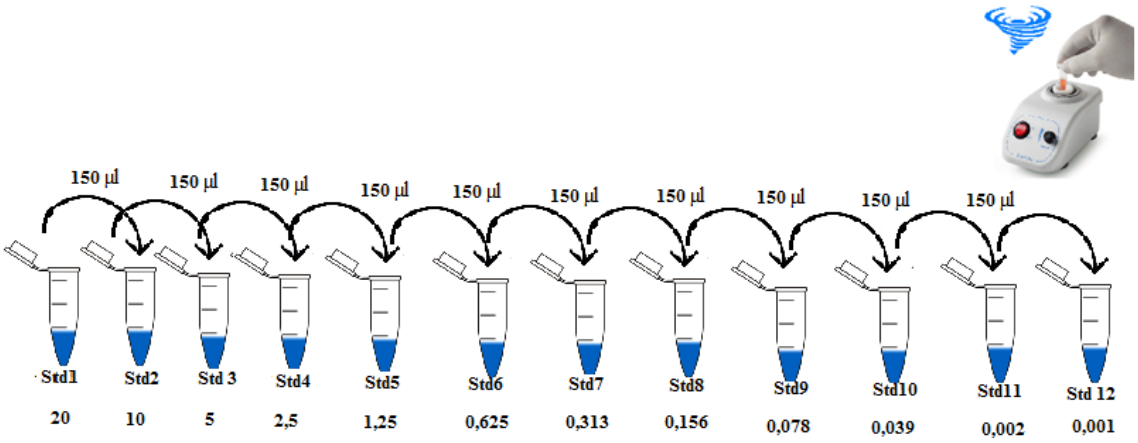

Figure 11. MIS Standards preparation illustration with each standard dilution in ng/ml.

Preparation of the microplate for MIS

The solutions were placed in the wells in the microplate; 50 µl TR+100 µl RB were added in the non-specific binding (NSB), 50 µl TR + 50 µl AB + 50 µl RB added in the maximum binding (B0), 50 µl TR + 50 µl AB + 50 µl Std were added in replicates in the standard wells (Std) and 50 µl TR + 50 µl AB + 50 µl of the samples (P) were add in replicates.

The microplates were cover with a plastic cover plate and aluminium and incubated overnight at 4 ºC and agitation 150 r.p.m. After the incubation the plates were washed 3 times in the Microplate washer and the last Ellman solution was prepared. To prepare

the Ellman 1760 µl of pure water was added with 440 µl of Ellman and added 200 µl in all the wells and more 5 µl of TR was added in the total activity (AT) wells. The microplates were covered again with a plastic cover and aluminium and incubated over night at 22ºC with agitation of 150 r.p.m.

3.7.5 ELISA assay

In order to obtain the results regarding the hormone steroids, the microplates were introduced in the microplate reader, set at 415 absorbance. The amount of transmitted light will be related to the concentration of the hormone of interest in ng/ml.

3.8 Histological analysis

During the last day of sampling 23 of May (Day 19), one fish from each treatment was killed (n=3) in order to extract the gonads for histological evaluation of the development of the germ cell in the testes. The testes were cut in small fractions of the center part and fixed in a 4% formaldehyde; 1% glutaraldehyde-buffered saline (McDowell and Trump, 1976) for later examination. The gonads were dehydrated in a 70 to 96% ethanol series of one hour and embedded in glycol methacrylate resin (Tecnovit 7100, Heraeus Kulzer, Germany). The resin with the gonads became hard blocks which were cut into 4 µm sections with a microtome (Leica RM 2245, Germany). Slides were stained with Azure II (Sigma, Germany) and Basic Fuchsin (Polysciences, USA) according to Bennett *et al.*, (1976). The slides with the sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

3.9 Statistical analysis

The obtained data for the sperm evaluation (motility (%), motility duration (min), density (spermatozoa/ml) and survival days after short storage, the sperm analysed with CASA (motility (%), VCL- µm/s, VAP- µm/s and VSL- µm/s) and the hormone steroids in ng/ml (T - ng/ml, 11-KT- ng/ml and MIS-ng/ml) were analysed using a two way ANOVA, with time (Day 0, Day 2, Day 5, Day 12 and Day 19) and treatment (Cntrl, Inj and EVAc) as the factors and Duncan post-hoc test. Sigma Stat was used for all statistical analysis.

4 RESULTS

4.1 Sperm analysis

The spermiation condition of the fish showed that there were significant differences among the treatments (ANOVA, $P < 0.001$). The fish treated with EVAc implants exhibited higher spermiation index than the other groups during the experimental period, which indicates that more fish that were treated with EVAc implant express easily sperm from the testes and consequently had more sperm volume. The treatment with GnRHa implants (EVAc) presented significant difference from the other treatments (Cntrl and Inj) and kept the higher conditions over the course of the experiment.

ANOVA TABLE	DF	P
Day	4	0,264
Treat	2	<0,001
Day x Treat	8	0,162
Residual	60	
Total	74	

Treat	P<0,050
EVAc vs. Cntrl	Yes
EVAc vs. Inj	Yes
Inj vs. Cntrl	No

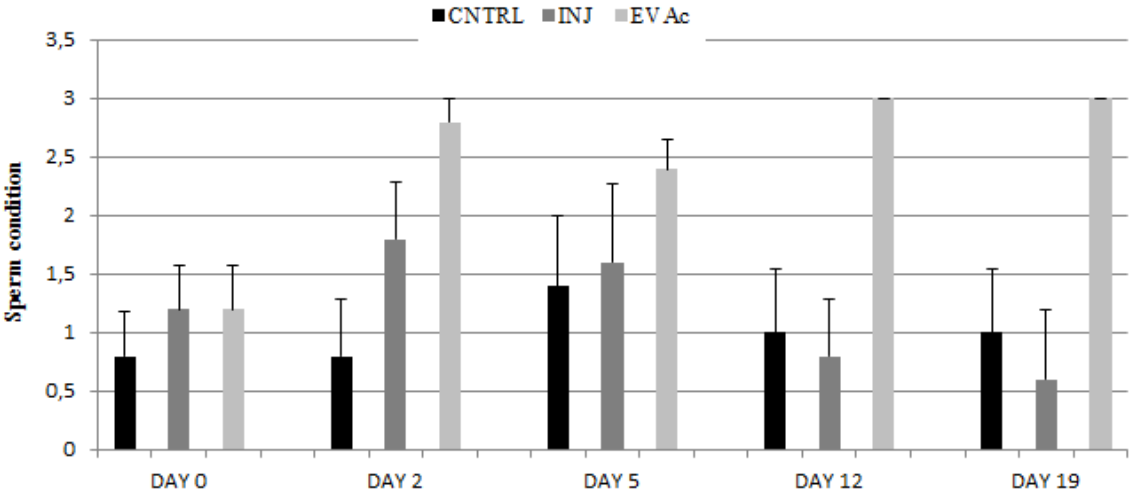

Figure 12. Sperm condition for the different treatments (Mean +S.E.M); Control (Cntrl), Injection with GnRHa (Inj) and Implant with GnRHa (EVAc) during the days of sampling. The two - way ANOVA shows the significant difference between the treatments (Treat) P < 0,001. The table with the treatments (Treat) shows the analysis of variance, Duncan`s new multiple rage test, $P < 0.05$.

The duration of sperm motility was a little more than 4 minutes as the longest survival time for the sperm of meagre during the experiment. The figure shows that there were no significant differences among the three treatments in duration of the sperm motility.

ANOVA TABLE	DF	P
Day	4	0,182
Treat	2	0,228
Day x Treat	8	0,188
Residual	31	
Total	45	

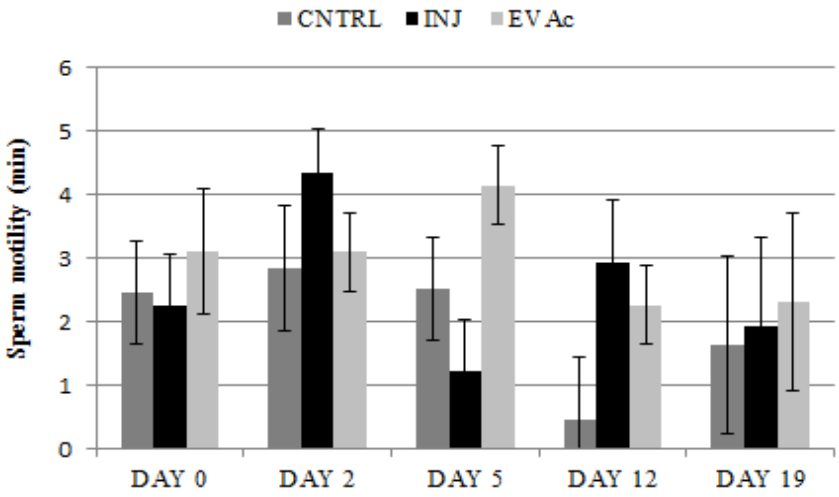

Figure 13. Sperm motility duration in minutes from the different treatments (Mean +S.E.M), Control (Cntrl), Injection (Inj) and Implant (EVAc) at different days of sampling during the spermiation period. Two - way ANOVA $P < 0.05$.

The results of the sperm motility percentages are shown in figure 14. Statistical analysis of the results shows that no significant difference was obtained among treatments. However in Day 5 there were significant difference compared to Day 0 and Day 19. The Day 5 also presented the lowest values during the time of the experiment and the EVAc treatment showed a trend to have always the highest values.

ANOVA TABLE	DF	P
Days	4	0,002
Treat	2	0,062
Days x Treat	8	0,354
Residual	31	
Total	45	

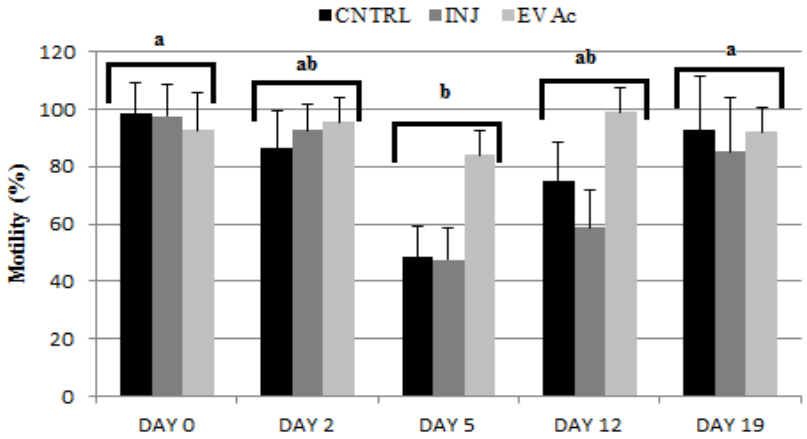

Figure 14. Sperm motility percentage for the different treatments (Mean +S.E.M); Control (Cntrl), Injection with GnRHa (Inj) and Implant with GnRHa (EVAc) during the days of sampling. The two- way ANOVA indicate the existence of a significant difference between the days (Days) of sampling (P = 0.002). The different letters indicate the means that were significant different between the days of sampling (analysis of variance, Duncan`s new multiple rage test, $P < 0.05$).

In general there was no effect of the treatment in the density, with an exception between the treatments Control and Injection where the Control group exhibited higher density mean (26.87 x 10^9 spermatozoa ml $^{-1}$) than the injection that presented the lowest means for the sperm density (18.89 x 10^9 spermatozoa ml $^{-1}$) .

ANOVA TABLE	DF	P
Days	4	0,354
Treat	2	0,029
Days x Treat	8	0,147
Residual	30	
Total	44	

Treat	P<0,050
Cntrl vs. Inj	Yes
EVAc vs. Cntrl	No
EVAc vs. Inj	No

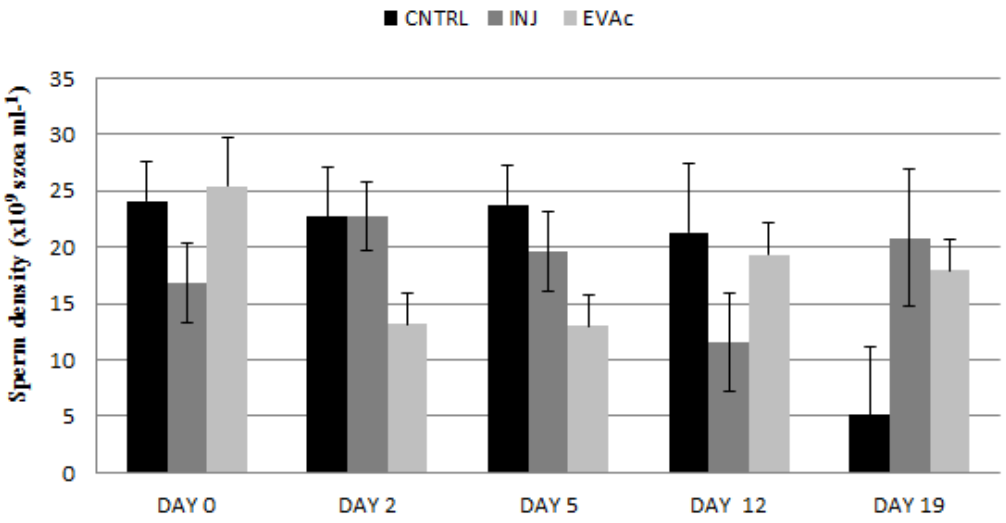

Figure 15. Sperm density for the different treatments (Mean +S.E.M); Control (Cntrl), Injection with GnRHa (Inj) and Implant with GnRHa (EVAc) during the days of sampling. The two- way ANOVA indicated the existence of a significant difference between the treatments (Treat) P = 0.029. The table with the treatments (Treat) indicates the analyses of variance, Duncan`s new multiple rage test, $P < 0.05$.

The milt from meagre presented a maximal survival of 5 days when stored at 4C. There were no significant differences between the treatments in the mean duration of sperm survival.

ANOVA TABLE	DF	P
Days	4	0,086
Treat	2	0,635
Days x Treat	8	0,746
Residual	31	
Total	45	

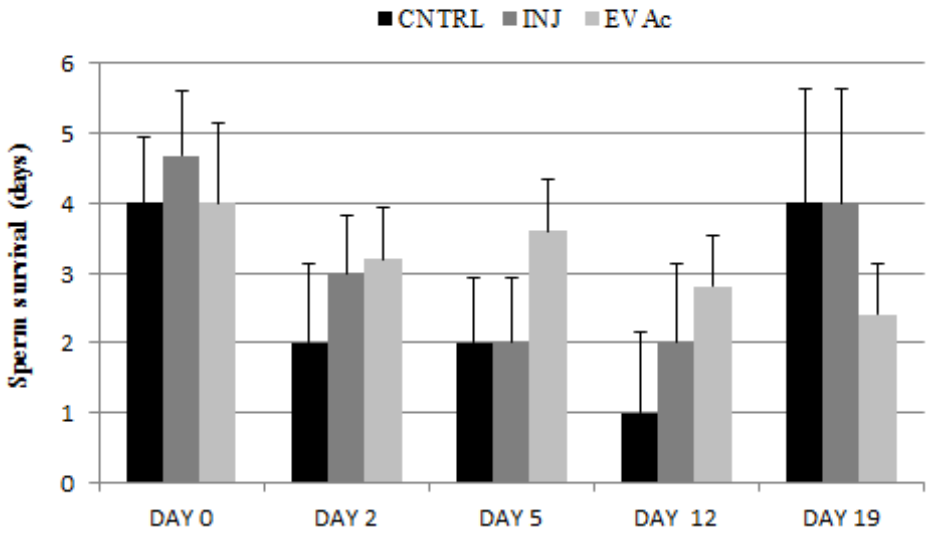

Figure 16. Sperm survival days from the different treatments (Mean +S.E.M), Control (Cntrl), Injection (Inj) and Implant (EVAc) at different days of sampling during the spermiation period of meagre (two- way ANOVA $P < 0.05$).

4.2 CASA Sperm analysis

For the parameters analyzed with the software CASA using sperm mix with NAM (data not shown) and fresh sperm (Figure 17) there were no significant differences among the three treatments in sperm motility (%), VCL, VAP and VSL.

Motility (%) CASA ANOVA TABLE	DF	P
Day	4	0,881
Treat	2	0,786
Day x treat	8	0,73
Residual	20	
Total	34	

VCL ANOVA TABLE	DF	P
Day	4	0,252
Treat	2	0,412
Day x Treat	8	0,708
Residual	20	
Total	34	

VAP ANOVA TABLE	DF	P
Day	4	0,221
Treat	2	0,293
Day x treat	8	0,758
Residual	20	
Total	34	

VSL ANOVA TABLE	DF	P
Day	4	0,547
Treat	2	0,453
Day x Treat	8	0,873
Residual	20	
Total	34	

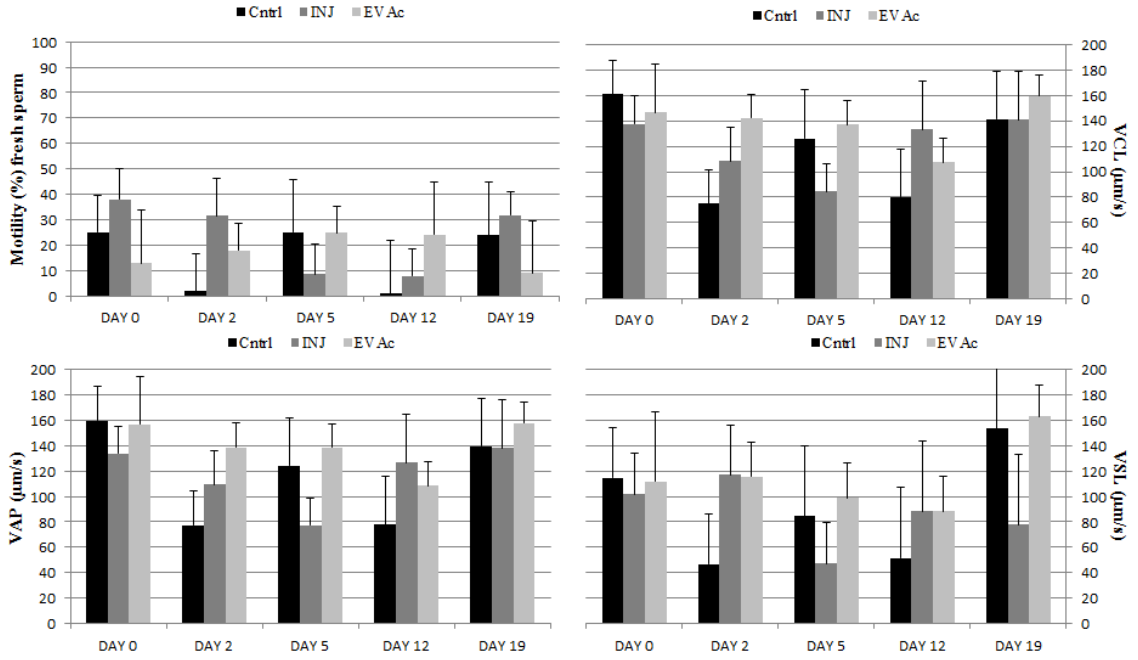

Figure 17. Sperm motility percentage analysed by CASA using fresh sperm from the different treatments (Mean +S.E.M); Control (CNTRL), Injection (INJ) and Implant (EVAc) at different days of sampling during the spermiation period (two – way ANOVA $P < 0.05$). Sperm curvilinear velocity (VCL) analysed by CASA using fresh sperm from the different treatments (Mean +S.E.M); Control (CNTRL), Injection (INJ) and Implant (EVAc) at different days of sampling during the spermiation period (two- way ANOVA $P < 0.05$). Sperm average path analysed by CASA using fresh sperm from the different treatments (Mean +S.E.M); Control (CNTRL), Injection (INJ) and Implant (EVAc) at different days of sampling during the spermiation period (two -way ANOVA $P < 0.05$). Sperm straight line velocity analysed by CASA using fresh sperm from the different treatments (Mean +S.E.M); control (CNTRL), injection (INJ) and implant (EVAc) at different days of sampling during the spermiation period (two-way ANOVA $P < 0.05$).

4.3 Hormone steroids

The hormone steroid testosterone (T), presents a statistical difference between the fish treated with EVAc and Control. Significant differences were also obtained between the Day 2 of treatment in comparison with all the other days of sampling. Furthermore, the Day 5 also exhibits a significant difference compared with all the days. The group treated with EVAc exhibited higher levels of testosterone during all days of experiment and the group Injection had the highest levels in Day 2 (Figure 18).

ANOVA TABLE	DF	P
DAY	4	<0,001
Treat	2	0,008
Day x Treat	8	0,003
Residual	59	
Total	73	

Treat	P<0,050
EVAc vs. Cntrl	Yes
EVAc vs. Inj	No
Inj vs. Cntrl	No

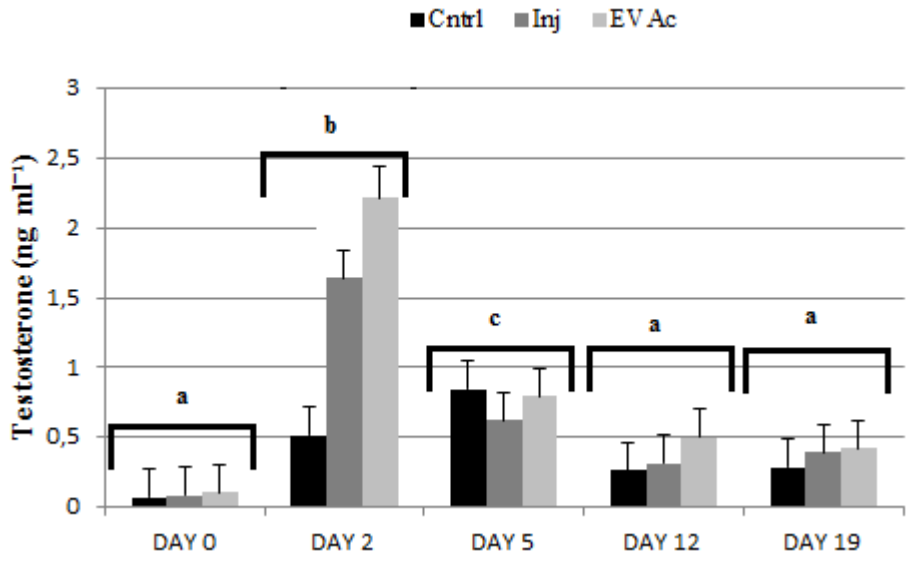

Figure 18. Testosterone plasma levels (ng ml $^{-1}$) from the different treatments (Mean +S.E.M); Control (CNTRL), Injection (INJ) and Implant (EVAc) at different days of sampling during the 20 days of experiment. The two- way ANOVA indicated statistically differences in the Day of experiment ($P < 0.001$) and between the treatments ($P = 0.008$) also a significant interaction between the Days and Treatment ($P = 0.003$). Different letters indicate means that were significant different between the Days of sampling (analysis of variance, Duncan`s new multiple rage test, $P < 0.05$).

The levels of 11-KT demonstrated that there were significant differences among the three treatments over the course of the study (two - way ANOVA P <0.001). Statistical significances were also presented for the Days of experiment, higher values were presented in Day 2 but Day 5 also showed significant differences. The fish treated with EVAc exhibited higher values than the other treatments during the entire experiment.

ANOVA TABLE	DF	P
Day	4	<0,001
Treat	2	<0,001
Day x Treat	8	0,311
Residual	59	
Total	73	

Treat	P<0,050
E vs. C	Yes
E vs. I	Yes
I vs. C	No

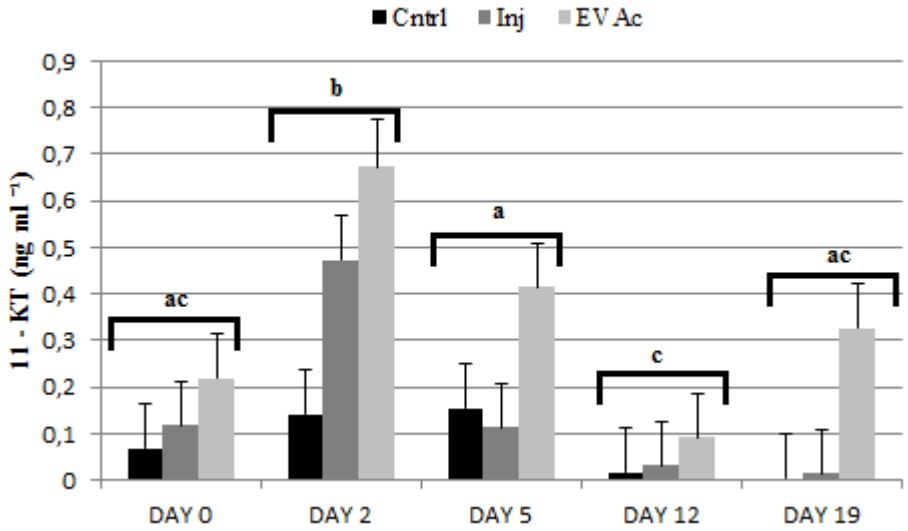

Figure 19. 11 - ketosterone plasma levels (ng ml $^{-1}$) from the different treatments (Mean +S.E.M); Control (Cntrl), Injection (Inj) and Implant (EVAc) at different days of sampling during the spermiation period of meagre. The two-way ANOVA indicated a statistically significant difference between the Days (P< 0.001) and between the Treatments (P <0.001). Different letters indicate means that were significantly different between the Days of experiment (analyses of variance, Duncan`s new multiple rage test, P < 0.05).

The data in figure 20 shows the T and 11-KT hormonal profile in meagre treated with GnRHa, Injection and implant (EVAc) and the Control. In Day 2 the levels of testosterone (T) and 11 ketostesterone (11- KT) were the highest for the fish treated with GnRHa while the Control presented higher levels compared with the other groups in the Day 5, even though in lower standard. In Day 12 the fish treated with EVAc had higher levels of T while 11-KT remained low, with the same levels for all the treatments. For the last day of experiment (Day 19) the levels of T had equal values while the 11-KT increased just for the EVAc treatment.

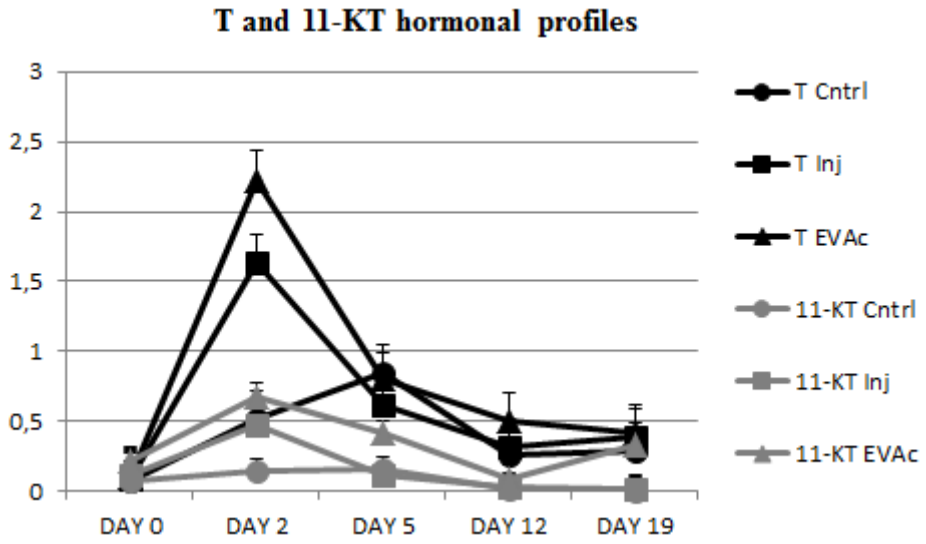

Figure 20. Hormonal profile of plasma levels of Testosterone (T) and 11 – Ketotestosterone (11-KT) (ng ml $^{-1}$) from the different treatments (Mean +S.E.M); Control (Cntrl), Injection (Inj) and Implant (EVAc) at different days of sampling over the course of the experiment of meagre.

The maturation inducing steroid (MIS) (Figure 21) showed no significant differences in Treatments and Days of sampling (two-way ANOVA $P < 0.05$). In Day 2 there was a slight rise in the serum levels of MIS.

ANOVA TABLE	DF	P
Day	4	0,437
Treat	2	0,555
Day x Treat	8	0,759
Residual	59	
Total	73	

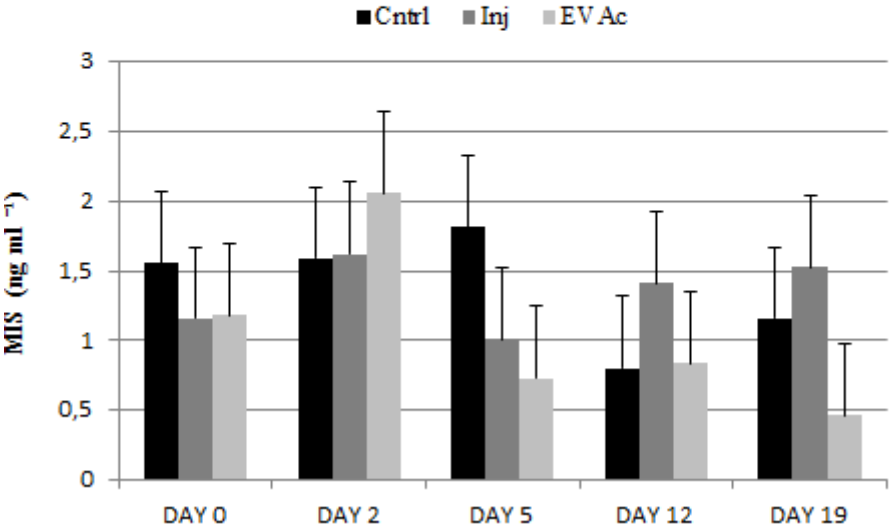

Figure 21. Steroid 17-20ß-dihydroxy-4-pregnen-3-one (17,20bP) or MIS plasma levels from the different treatments (Mean +S.E.M); Control (Cntrl), Injection (Inj) and Implant (EVAc) at different days of sampling during the experiment period (two - way ANOVA $P < 0.05$).

4.4 Histology

The histological sections of the test from the Control, Injection and EVAc treatments were divided in 3 images, each one exemplified different areas of the testis. The central area of the testis (A1, A2 and A3), the middle of the testis (B1, B2 and B3) and the periphery (C1, C2 and C3) in different magnifications. For the histological sections from the fish representing the Control group (Figure 22), in the central area of the testis (A) were identified 4 stages of development of germ cells along the connective tissue: spermatogonia (Sg1, Sg2) and spermatocytes (Sc1, Sc2) and the deferent vasa were full filled with spermatozoa. In the middle of the testis (B) there were differences, just a few germ cells developing (Sg1, Sg2), a connective tissue thicker than the central area but the deferent vasa remained full filled with spermatozoa. The periphery of the testis presented higher density of first stages of germ cells, mostly Sg1 and Sg2 with a few Sc covering the limit of the periphery area, the connective tissue had an intermediate

thickness but still thinner than the middle part. There were some empty spaces in the deferent vasa with no spermatozoa, showing that the fish no longer would produce spermatozoa. The control fish exhibited the aspect of the testis from a fish that was by the end of it reproductive season and starting the regressing phase, increasing continuous the proliferation of Sg1 as a consequence. The fish treated with GnRHa Injection (Figure 23), showed a thick connective tissue in the central area of the testis (A), a few Sg1 and Sg2 and the deferent vasa mostly full filled with spermatozoa (Sz) and some minor areas with no spermatozoa viewing empty spaces. However in the middle area (B) the connective tissue is thin but with a little bit more germ cells in development (Sg1 and Sg2), spermatozoa in the deferent vasa and a few exceptions with empty spaces. For the last part, in the periphery of the testis the connective tissue remained thin but with a few more germ cells in development (Sg1 and Sg2), the spermatozoa were taking place in most of all the deferent vasa. The fish with Injection treatment presented the condition of a testis that is finishing the spawning season and stopped to develop spermatozoa, but still with milt. In the histological photos of the testis from the fish treated with GnRHa implant (EVAc) (Figure 24) the connective tissue was thin in the central area and all the stages of germ cells were identified, Sg1, Sg2, Sc1, Sc2 and the spermatids (St). In the middle part of the test Sg1 and Sg2 were identify and the connective tissue was a bit thicker. The periphery of the testis presented a thin connective tissue and germ cells along (Sg1 and Sg2), also spermatids (the cells that would acquire capacitation to became spermatozoa). In all the 3 parts of the test that was photographed there was spermatozoa and in the position come out. The fish from EVAc treatment showed a testis that represents a fish in the spawning season, and would continuous to produce sperm.

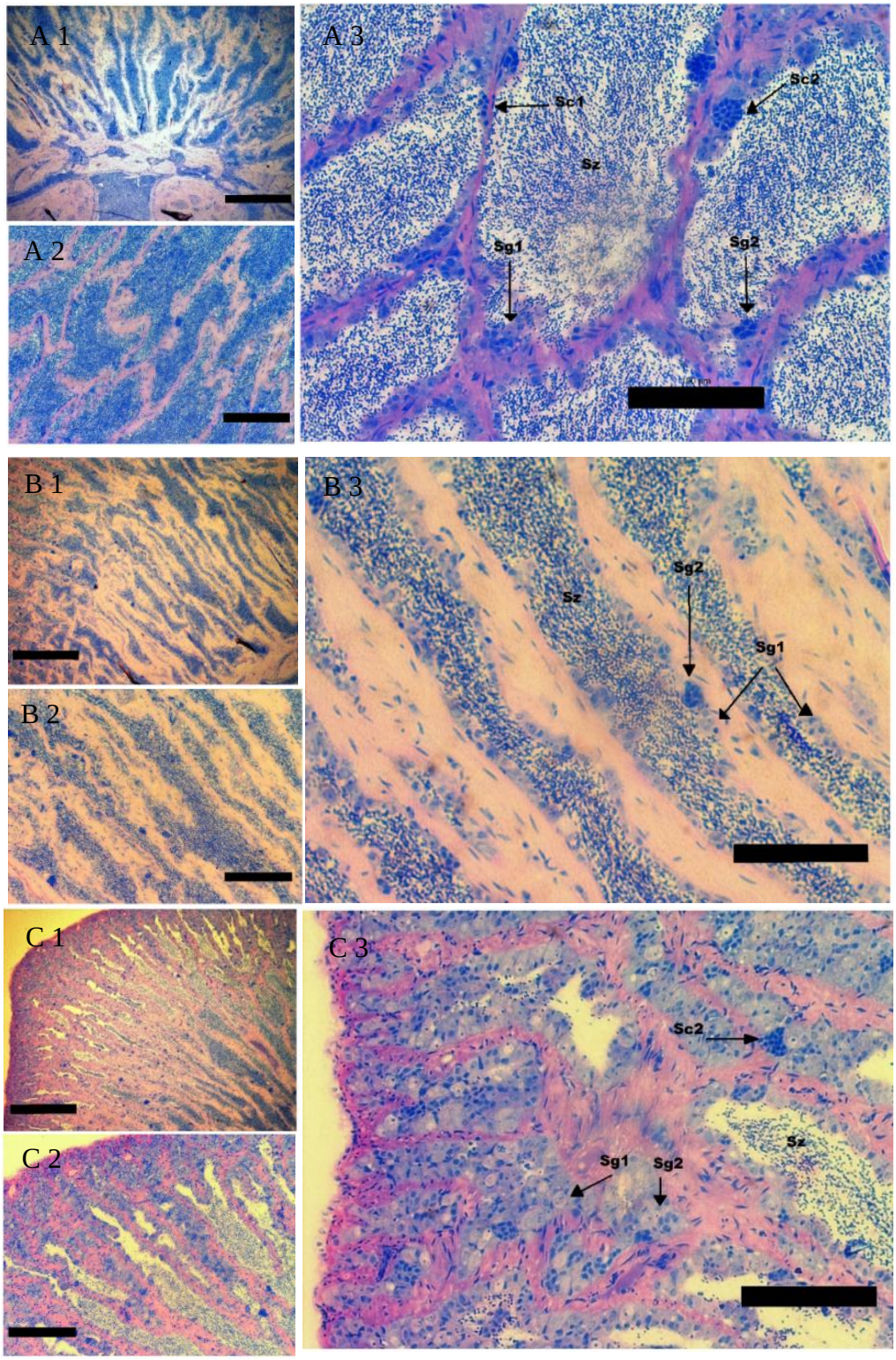

Figure 22. Histological section of a test from meagre in the Control group after 20 days of experiment. The section (A) it is referent to the central part of the testes mainly full of spermatozoa (Sz), with some cells of spermatogonia (Sg1, Sg2) and spermatocytes (Sc1, Sc2). Section (B) of the middle part of the testes with a thick connective tissue and the deferent vasa filled with spermatozoa (Sz) a few spermatogonia (Sg1, Sg2) . Section (C) of the periphery section of the testes mostly with early spermatogenesis (Sg1, Sg2) and empty areas with no spermatozoa. The bars in A1, B1 and C1 represents 500 µm, the bars in A2, B2 and C2 represents 200 µm and C1, C2 and C3 100 µm.

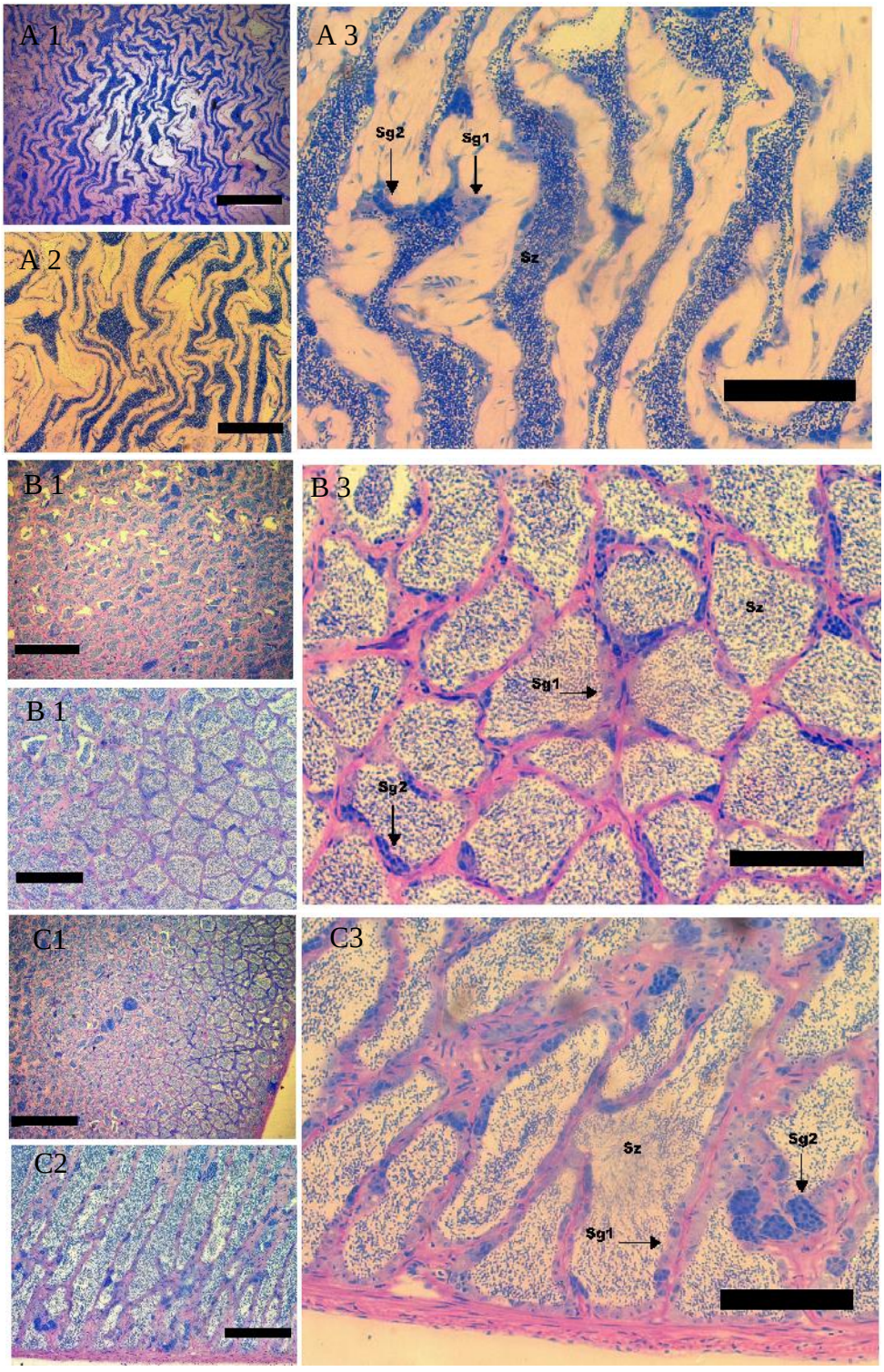

Figure 23. Photograph of histological sections of a test from a fish Injected (Inj) with Gorham after 20 days of experiment. Section (A) shows the central part of the test mainly full of spermatozoa (Sz), with some cells of spermatogonia (Sg1, Sg2), connective tissue thick. The Middle part of the testes (B) presented deferent vasa filled with spermatozoa (Sz) and a few Spermatogonia (Sg1, Sg2) . Periphery section (C) of the test with some spermatogoia (Sg1, Sg2) and some areas with no spermatozoa. Sperm condition S0. The bars in A1, B1 and C1 represents 500 µm, the bars in A2, B2 and C2 represents 200 µm and C1, C2 and C3 100 µm.

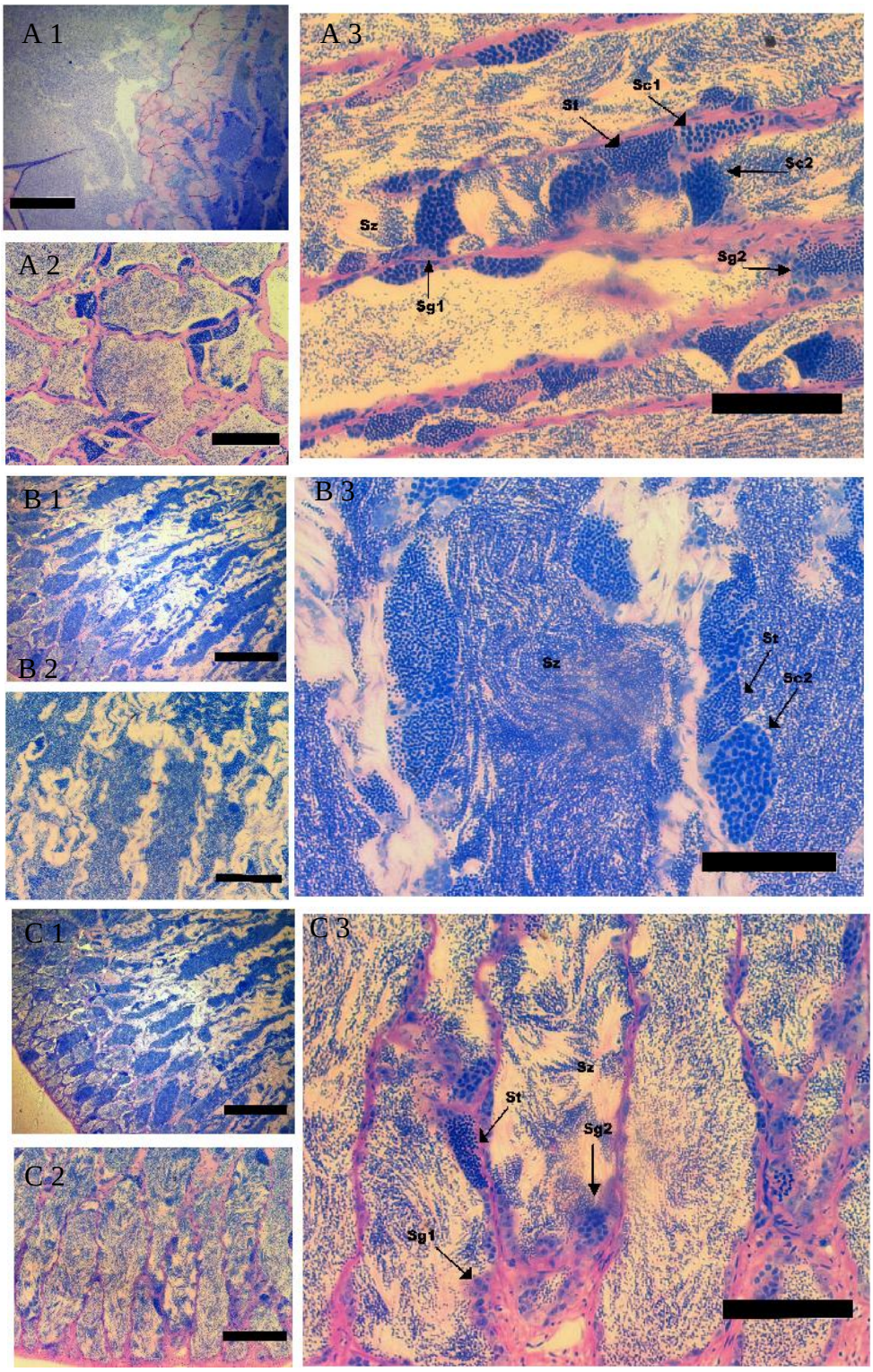

Figure 24. Photograph of histological sections of a teste from a fish treated with implant (EVAc) Gorham after 20 days of experiment. Section (A) from the central part of the test with all stages of spermatogenesis and spermiogenesis, spermatogonia (Sg1, Sg2) spermatocytes (Sc1, Sc2) spermatids (St) and spermatozoa (Sz), conective tissue thick. The Middle part of the test (B) with deferent vasa filled with spermatozoa (Sz), spermatocytes (Sc2) and spermatids (St). (C) Periphery section of the testes, thin connective tissue with some spermatogonia (Sg1, Sg2) spermatids (St) and spermatozoa (Sz) ready to be release. Sperm condition S3. The bars in A1, B1 and C1 represents 500 µm, the bars in A2, B2 and C2 represents 200 µm and C1, C2 and C3 100 µm.

5. DISCUSSION

The Knowledge in the reproductive strategy of each species is the key to achieve successful and efficient gametes production. Aspects such as temperature, season or month of spawning, frequency of batch, fecundity and fertilization are very important to produce good quality of fish. Gametes production can be a limit factor in quantity and quality of commercial hatcheries (Mylonas *et al.*, 2010; 2013b). Nevertheless, a major issue for aquaculture industry is the unpredictable and reduced amount and quality of milt volume in some species. Sperm quality is an essential variable in aquaculture broodstock management which has impact in the fertilization, production of viable eggs and larvae (Cabrita *et al.*, 2014). Therefore studies in fish reproduction are helpful for improving broodstock management but also to develop suitable methods to enhance spermiation (Mañanós *et al.*, 2002).

The results for enhancement sperm production in *Argyrosomus regius* support a prolongation of spawning for the fish treated with GnRHa implants (EVAc), since all the fish produced sperm in condition S3 until the end of the experiment. This method has great acceptance once that induce the spermiation for long period when compared to the injection treatments and also avoid handling the fish consecutive times (Mylonas *et al.*, 2016a). The treatment with GnRHa Injection seemed to have an effect in the sperm condition for 2 days after application, decreasing gradually over the pass days. However, when the second injection was applied in Day 12, the fish presented the lowest values after 5 days (Day 19). These observations suggest that the GnRHa Injection within 5 days does not present any positive effect concerning the sperm production and volume for meagre during this experiment. It is known that single injection with GnRHa increases the sperm volume in a short term effect, for a few hours or a few days (Mylonas *et al.*, 2016a). The same results for expressible milt were found for the European sea bass (*Dicentrarchus labrax*), when a single injection induced the sperm production for 3 days, but dramatically decrease afterwards (Rainis *et al.*, 2003). Moreover, for the golden rabbitfish the injection induced an increase of the sperm volume for 24 hours and after 48 hours the spermiation was already similar to the control group (Garcia, 1991). Regarding this experiment, the Control group had the best sperm condition in the Day 5 of experiment, the middle of the spawning season. However the sperm motility percentage for this day exhibited the lowest value. Several studies confirm that changes in the sperm quality occurs naturally over the course of the

reproductive season (Billard *et al.,* 1995; Fauvel *et al.,* 2010; Rainis *et al.,* 2003; Schiavone *et al.,* 2012). In meagre reared in Italy, Schiavone *et al.* (2012) found the best sperm quality parameters during middle of June while the worst was in the early June. Nevertheless changes in the sperm quality in captive fish can also be related with the stress imposed by culture conditions (Billard *et al.,* 1995; Schreck, 2010). The motility minutes were not affected by the treatments although the motility percentage (%) showed a difference in the Day 5. The fact could also be explained by a technical error, the sperm could be contaminated with water or urine which leads to a decrease in the sperm motility and reduction of ATP intracellular store (Poupard *et al.,* 1998). Meagre are known to produce low milt volume in captivity (Mylonas *et al.,* 2013a), and sperm collection can be difficult when the fish does not present high sperm conditions (Spermiation index $\geq$ 2). However for meagre the sperm motility parameters and survival seemed not be affect by the hormonal treatments here. In a similar study with meagre the above related parameters did not present any change regarding the hormonal treatment (Mylonas *et al.,* 2016b). Other species such as *Osmerus eperlanus* (Król *et al.,* 2009) and the white bass (*Morone chrysops*) (Mylonas *et al.,* 1997) also did not present any difference in the sperm motility and survival when fish were treated with GnRHa. The increase production of sperm in volume in some cases can affect the sperm motility, but it depends on what species and the characteristics that presents (Mylonas *et al.,* 2016a).

One of the functions of GnRHa hormonal treatment in the fish physiology is an increase of seminal fluid, pH and hydration of the testis, which is related with the MIS steroid. This process is very important for the fish reproduction, once that gives capacity to the sperm to fertilize (Cabrita *et al.,* 2014 ; Mylonas *et al.,* 2010). The number density of spermatozoa normally is not affect by hormonal treatments, even though in some cases this increase in the seminal fluid can be positive, especially to species that presents poor milt production and thick sperm in captivity such as the smelt (*Osmerus operlanus*) (Król *et al.,* 2009), golden rabbitfish (*Siganus guttatus*) (Garcia, 1991) and the halibut (*Hippoglossus hippoglossus*) (Vermeirssen *et al.,* 2004). Species with thick or high concentration of sperm present low fertilization rates once that the sperm cannot disperse well in the water (Mylonas *et al.,* 2016a). In this experiment the fish treated with GnRHa Injection exhibited statistic higher density than the Control in the Day 19, most probably due to the low number of individuals sampled (n=1) for this day.

CASA sperm analysis

There was no effect of the GnRHa treatments on the quality of sperm tested with CASA, also the non-activate medium Leibo did not exert any difference in the quality of the sperm. The CASA method commonly used for fish sperm track analysis is a modification from computer sperm analysis of human sperm. Therefore to use this method for fish sperm motility it requires some adaptations and most of the present systems for CASA are complex and expensive. The sperm of most teleost fish differ from the mammals aspects in trajectories, activation mode and time and the fact that teleost fish spermatozoa do not present acrosomes and access the egg through micropyle (Kime *et al.,* 2001). However, the most important advantage of CASA used in this experiment is its analyses without complex equipment and expensive software. The CASA method can be useful for a range of purpose, such as influence of environment pollutants, optimize conditions for artificial fertilization, broodstock selection and experiments with cryopreservation and sperm storage (Cabrita *et al.,* 2014; Kime *et al.,* 2001; Pavlov, 2006). Although no significant improvement on motility parameters was determined with CASA software, the system proved to be an accurate method to determine motility characteristics in meagre sperm.

Hormone Steroids

Testorerone (T) presented the highest levels after 2 days of treatment with GnRHa (Inj and EVAc),but Day 5 also had a significant difference. Notwithstanding within the Control group, the highest levels of T and sperm condition were in Day 5. These results may show that testosterone exert an important function in the first meagre spermatogenesis. In another experiment that analysed the hormone profile of meagre during the first sexual differentiation and maturation, testoterone exhibited a key role in the sex differentiation of meagre (Schiavone *et al.,* 2012). Furthermore, the levels of 11-KT remained low for all the fish in the experiment. However, the fish treated with EVAc presented the highest plasma levels, which seems to be important to maintain the spermiation condition high. On the other hand the injected fish, after the second injection in Day 12 exhibited the lowest levels of 11-KT (Day 19) and also the lowest sperm volume. The concentration of the plasma levels of T and 11-KT presented in this experiment were in accordance with other study on the reproduction of meagre (Schiavone *et al.,* 2012) . However the present study showed T and 11-KT levels in the

same order of significance as in the experiment done previously by Mylonas *et al.* (2013b), but with higher concentrations. Gonadal growth and spermatogenesis, generally are associated with an increase in T and 11-KT. Nevertheless 11-KT is derived from T and can be considered a hormone that stimulates secondary sexual characters and spermatogenesis in a more physiological form, while T seems to have an important role in the early phases of the sex differentiation (Schiavone *et al.*, 2012). The GnRHa treatment in this experiment, produced a significant difference in the T and 11-KT plasma levels and the steroid T exhibited higher concentration than the 11-KT, which may explain that it was the first spawning season of this fish. Regarding the MIS, no significant differences were found and the levels remained higher than T and 11-KT hormone. Even so, it should be noted that the fish treated with GnRHa Injection exhibited the same levels of MIS in Day 2 and Day 19, but not the same as T and 11-KT, which corroborate the importance of the last two hormones. Additionally, the sperm characteristics of the group of treated with GnRHa Injection presented the highest sperm volume in Day 2 and the lowest in Day 19. The results lead to conclude that T and 11-KT are the main important hormones regarding the first spermiation in meagre, and confirm the same results found by Schiavone *et al.* (2012). The effect of GnRHa treatment in this species may stimulate the stored release of the FSH in the pituitary. The FSH is the main hormone that induces the increase of T and 11-KT, which are as previously mentioned are involved in the spermatogenesis, spermiogenesis and spermtiation (Mylonas and Zohar, 2009). In another experiment with GnHRa implant in rabbitfish (*Siganus guttatus*) during pre-spawning season, the mean levels of 11-KT and T were significant higher than the control group, confirming that GnRHa treatment induced the process of development and maturation of germ cells. (Komatsu *et al.*, 2006).

Histology

The results of histology in meagre testes demonstrated that the fish presented an asynchronous type of gonadal maturation, with the different stages of germ cells placing the different areas of the testes, exemplifying an unrestricted type of testes. Regarding the effect of treatments in the development of the germ cells, the histological results showed the developing stages of spermatogenic germ cells in the testes and sperm. The lack of spaces with no spermatozoa may lead to conclude that the sperm production stopped and, combined with the results of sperm condition S0, no milt came out from

the testes. Therefore, the histological images from the Implant group proved that the fish was in full spermiation with S3 condition and would continuous spermiating, once that exhibit all stages of spermatogenic germ cells, especially spermatids that will become spermatozoa (Prista *et al.,* 2014; Schiavone *et al.,* 2012). A possible prolongation of spermiation is justified by the different stages of germ cells presented in the gonads noticed in the fish treated with GnRHa Implant.

6 CONCLUSION

In conclusion, the treatment with GnRHa injection showed a short period of 2 days of effect regarding enhance the sperm production in meagre. Implant with GnRHa proved to enhance the sperm production in meagre for a prolonged time, once that induced the development and maturation of spermatogenic germinal cells, inducing spermiation and the extraction of the milt in higher volume. The GnRHa implant in meagre demonstrated that the treatment can be useful to induce the spermiation in the beginning of spawning season. Furthermore, the GnRHa treatment did not affect the quality of the sperm, and CASA demonstrated to be an important tool for measure precisely the sperm quality parameters required for improve methods on sperm quality.

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Appendix 1

Dilution for sperm density:

1º - 10µl sperm diluted in 200 µl saline
2º - 10µl solution diluted in 1000µl saline
} total of dilution 2121

CASA solutions:

1) Non activate medium (NAM) LEIBO:
2) Activate medium: Bovine Serum Albumin (BSA) + seawater from the fish tank(SW)

CASA dilutions:

1) 50 µl NAM + 25 µl fresh sperm
2) 2ml BSA + 18ml SW

CASA record:

1) 4-9 µl NAM + 99µl Activate medium
2) 1-2 µl fresh sperm + 99 µl Activate medium
} 1µl of each solution add on Leja

Appendix 2

ELISA solutions:

Coating of microtiter plates

1. Monoclonal antibody (monoclonal anti-rabbit IgG, Sigma, R-1008, 0.2 ml).
2. Reconstitution in 5 ml PPB (Potassium Phosphate Buffer) 0.05 M (20 x i M), concentration 1 mg/ml.

For 20 microplates:

Place the antibody in 10 plates and then we use the same antibody in another 10 plates.

- 198 ml PPB 0.05 M
- Addition of 2 ml monoclonal antibody 1 mg/ml to get a concentration of 10 μg/ml.
- Add in 10 plates of this antibody concentration (200 μl/ well).
- Leave plates at 20^{o} C overnight without stirring.
- The next day use a multichannel pipette to take out the antibody. Use a usual pipette to take out the remaining antibody from each well. Add 300 μl RB in each well.
- Add antibody recovered from the 10 plates in 10 new plates (200 μl/ well).
- Leave the new plates at 20^{o} C– the old ones with the RB at 4^{o} C.
- Without taking out the antibody from the 10 last plates add Saturation buffer (100 μl/ well).

REACTION BUFFER (RB)

- 100 ml potassium phosphate buffer 1M
- 0.1 g NaN_3
- 23.4 g NaCl
- 0.37 g EDTA
- 1g BSA (Bovine serum albumin)
- 1:1 distilled water.

WASHING BUFFER

- 10 ml potassium phosphate buffer 1M
- 0.5 ml Tween 20.
- 1:1 with distilled water.

ELLMAN'S REAGENT

- 20 ml potassium phosphate buffer 0.5 M.
- 215 mg DTNB (5'5' dithio – bis – nitrobenzoic acid)
- 200 mg acetyl thiocholine

Aliquots of 440 µl preserved and protected from light at – 20^{o} C.

For each assay dilute one aliquot in 21.560 ml dd H2O before use. Add 200 µl in each well. Protect from light.

TRACER (0.5 UE/ml)

ANTIBODY (1/1000)

TESTOSTERONE ANALYSIS

STANDARD CURVE

Testosterone concentration in Std1 is 5 ng/ml.

Aliquots were at a concentration of 1 µg/ml.

11 – KETOTESTOSTERONE ANALYSIS

STANDARD CURVE

11-ketotestosterone in Std1 is 1 ng/ml.

Aliquots were at a concentration of 10 ng/ml.

MIS

STANDARD CURVE

Aliquots were at a concentration 3ng/ml.